Mesangial function and glomerular sclerosis in rats after unilateral nephrectomy

Grond, Joris; Schilthuis, M.S.; Koudstaal, J.; Elema, J.D.

Published in:
Kidney International

DOI:
10.1038/ki.1982.178

Kidney International, Vol. 22 (1982), pp. 338–343

Mesangial function and glomerular sclerosis in rats after unilateral nephrectomy

JORIS GROND, MARTEN S. SCHILTHUIS, JAN KOUDSTAAL, and JOB D. ELEMA

The Department of Pathology, University of Groningen, Groningen, The Netherlands

Mesangial function and glomerular sclerosis in rats after unilateral nephrectomy. To investigate the possible relationship between disturbance of mesangial function and segmental localization of glomerular sclerosis, five uninephrectomized male Wistar rats and five sham-operated controls received colloidal carbon intravenously. At 4 months 8.4 ± 2.5% of the glomeruli of the nephrectomized rats showed focal sclerosis. Glomeruli of nephrectomized rats contained significantly more carbon than glomeruli of controls. Glomeruli with focal sclerosis contained significantly more carbon than normal glomeruli in the same kidneys with a preferential tracer localization within the lesions. In another experiment carbon injections were given *before* surgery. At 4 months 12.6 ± 4.1% of the glomeruli of the nephrectomized rats showed focal sclerosis, an incidence not significantly different from that of the first experiment. Glomerular carbon content was equal in experimental and control rats and no preferential localization of the tracer within the lesions was found. From these results we conclude that the preferential localization of carbon in the glomerular lesions in rats nephrectomized *before* injection of carbon is caused by the increased delivery of tracer shortly after injection to those glomerular areas where sclerosis will develop at a later time. The development of focal sclerosis may be related to the local deposition of harmful substances from the circulation.

Fonctionnement mésangial et sclérose glomérulaire chez des rats après néphrectomie unilatérale. Afin d'étudier une éventuelle relation entre des anomalies du fonctionnement mésangial et la localisation segmentaire de la sclérose glomérulaire, cinq rats mâles Wistar uninéphrectomisés et cinq contrôles ayant eu un simulacre d'intervention ont reçu du carbone colloïdal par voie intraveineuse. Au bout de 4 mois, 8,4 ± 2,5% des glomérules des rats néphrectomisés avaient une sclérose focale. Les glomérules des rats néphrectomisés contenaient significativement plus de carbone que les glomérules des contrôles. Les glomérules avec une sclérose focale contenaient significativement plus de carbone que les glomérules normaux des mêmes reins, avec une localisation préférentielle du traceur dans les lésions. Dans une autre expérience des injections de carbone ont été faites *avant* la chirurgie. Au bout de 4 mois, 12,6 ± 4,1% des glomérules des rats néphrectomisés avaient une sclérose focale, cette incidence n'étant pas significativement différente de celle observée au cours de la première expérience. Le contenu en carbone des glomérules était identique chez les rats expérimentaux et contrôles, et il n'a pas été trouvé de localisation préférentielle du traceur dans les lésions. De ces résultats, nous concluons que la localisation préférentielle du carbone dans les lésions glomérulaires de rats néphrectomisés *avant* une injection de carbone est dûe à une augmentation de l'afflux du traceur peu de temps après l'injection dans les aires glomérulaires où la sclérose se développera ultérieurement. Le développement d'une sclérose focale pourrait être relié au dépôt localisé de substances délétères provenant de la circulation.

Received for publication December 18, 1981
and in revised form April 1, 1982

0085–2538/82/0022–0338 $01.20

Lesions in glomeruli of aging rats develop and bear great similarity to focal segmental glomerular hyalinosis and sclerosis (FSGHS) in humans. In contrast to FSGHS lesions in humans which develop first in the juxtamedullary glomeruli [1, 2], in rats both cortical and juxtamedullary glomeruli are affected equally [3–5]. This spontaneous disease in rats is preceded by the development of proteinuria [3, 4, 6] and accompanied by biochemical alterations characteristic of the nephrotic syndrome [6–8]. Among factors held responsible for this disease are functional overload [3, 4, 9, 10], amino acid toxicity [8, 11–13] and aging [3, 14, 15]. Female rats seem to be less susceptible to the disease than males [4, 5, 8, 14, 16]. The primary site of the histologic changes appears to be the glomerular mesangium [3–6, 11]. There is a frequent relationship of the early lesions with the vascular pole of the glomerulus [4, 6], and it was suggested in earlier studies that hemodynamic factors could be important in the pathogenesis of FSGHS in the rat [4, 10, 17].

In this study an attempt was undertaken to elucidate the reason for the typical segmental character of the FSGHS lesions. We provide data suggesting that a locally increased mesangial deposition of circulating substances may precede and eventually induce the development of FSGHS lesions.

Methods

Rationale of experimental design. To facilitate the appreciation of the reasoning behind the different experiments, the following rationale is provided. Details of each experimental protocol are given separately.

Unilateral nephrectomy was used as a means to accelerate the development of FSGHS in rats. Under these circumstances the development of FSGHS may be caused by an increased burden to the glomerular mesangium as a consequence of increased glomerular flow and pressure [4, 10, 17].

Colloidal carbon was chosen to study mesangial function [18, 19] since the particles are indigestible, taken up quickly by the mesangium, and retained for considerable time. Previously it was shown that mesangial functional characteristics were similar in superficial and deep glomeruli [18].

The relation between the development of FSGHS lesions and alterations in mesangial uptake and clearance was studied with two experiments. In experiment A colloidal carbon was injected immediately *after* unilateral nephrectomy. The rats were killed 4 months later when FSGHS was expected to be present. The relationship between localization of carbon and FSGHS was analyzed by light microscopy. If the development of the

FSGHS lesions and their segmental character was related to local disturbance of mesangial function, we expected to find a preferential localization of remaining carbon in the lesions.

However, different mechanisms may underlie an identical localization of carbon and FSGHS lesions. At the time of injection an increased delivery of carbon may have occurred to those glomerular areas where sclerosis will develop at a later time. Alternatively, mesangial clearance mechanisms in those areas may be disturbed as a result of functional changes induced by unilateral nephrectomy. Therefore, to ensure an undisturbed mesangial uptake of carbon, in experiment B colloidal carbon was injected *before* unilateral nephrectomy was done. The rats were killed 4 months later. If, in this experiment, a relation between localization of carbon and of FSGHS lesions was found again, the reason for this should be a disturbance of carbon clearance from that area. If, however, such a relation was not present, increased delivery of carbon immediately after injection to the area in which FSGHS lesions eventually were going to develop should be the explanation for a possible positive finding in experiment A.

Experiment A. Ten male Wistar rats aged 3 months and weighing 180 to 220 g were used. They were fed a normal rat chow with free access to tap water throughout the course of the experiments. Five rats underwent right-side nephrectomy, the others a sham operation. Two weeks after the operation, all animals received weekly intravenous injections of 30 mg of colloidal carbon per 100 g of body weight during a 3-week period (colloidal carbon for biological use C 11/1431[a], containing 100 mg of carbon per milliliter, Günther Wagner, Hanover, Germany). Four months after the start of the experiment, urinary protein excretion was measured by the biuret method in urine collected by housing the rats during 24 hr in metabolic cages. During this time they had access to water only. Subsequently, the rats were killed by decapitation. Cross sectional slices were cut from the kidneys at the level of the vascular pedicle, fixed in 8% buffered formalin and embedded in glycolmethacrylate. From each tissue block two consecutive 2-mμ sections were cut on a Sorvall-JB-4 microtome with 34-mm glass knives. One set of sections was stained with periodic acid-Schiff for light microscopic examination; the other set was left unstained and used for the measurement of the amount of mesangial carbon. The amount of carbon present in the glomerular mesangium was assessed semiquantitatively with a modular scanning image analyzer (Optomax, Ealing Beck, Ltd, Whatford, England). This system consists of a light microscope (LM) equipped with an optical input system (camera) which projects the LM picture on a "central processor unit" made of maximally 108.080 points and equipped with an image scanner. The LM picture presented to the "central processor unit" is visible on a video monitor. The area to be scanned is manually set and indicated by a frame on the monitor image. In our study this area was set so that each time it framed the glomerulus selected for counting. For the purpose of our study the threshold of sensitivity was chosen visually with the help of the video monitor in such a way that only carbon particles gave sufficient contrast for detection by the scanner. Possible contribution of non-carbon contrast was excluded by the use of unstained sections. The method is a point counting method in which the total number of points covered by a contrasted area is counted and serves as a measure of the total area of carbon present in the glomerulus. The validity and reproducibility of this method for our purpose have been described elsewhere [19]. The principles of point counting and image analyzing methods have been discussed in detail by Weibel [20]. In each kidney measurements were made of 20 consecutive glomeruli sectioned through their largest or nearby largest diameter moving through the cortex from surface to medulla and vice versa. Mean count number per glomerulus was taken as a measure of mesangial carbon content of that kidney and the mean count of the five kidneys as the value for the group. For a more detailed analysis glomeruli were grouped in classes of increasing count number with a class range of 30 counts. In the kidneys of nephrectomized rats the distribution of 108 normal and 52 diseased glomeruli over the different count classes was also determined separately.

Experiment B. Ten male rats received a weekly intravenous injection of 30 mg of carbon per 100 g of body weight during 3 weeks. Two days *after* the last carbon injection five rats underwent right-side nephrectomy, the others a sham operation. Mesangial carbon amount reaches a maximum at about 24 to 32 hr after intravenous injection and gradually decreases thereafter over several months [18]. Four months after the operation urinary protein excretion was measured during a 24-hr period and all animals were killed. Kidney tissue and semiquantitative measurement of mesangial carbon content in the different groups and types of glomeruli were processed similarly to that in experiment A.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney U test or Student's t test. A probability value less than 0.05 was regarded as significant. Values are expressed as means. Where appropriate, the standard error of the mean (SEM) is given.

Results

Experiment A. Protein excretion 4 months after unilateral nephrectomy was 55.7 ± 9.0 mg/24 hr and after sham operation 12.8 ± 2.9 mg/24 hr. This difference was significant. At sacrifice 8.4 ± 2.5% of the glomeruli in the remaining kidneys of unilaterally nephrectomized rats showed characteristic FSGHS lesions consisting of segmental subendothelial deposition of hyalin eosinophilic material with an increase of mesangial matrix and capillary wall wrinkling including collapse and adhesions to the Bowman's capsule. No FSGHS lesions were found in the control group. The mean carbon content of the glomeruli in the control group was 31.1 counts per glomerulus and in the group with unilateral nephrectomy 80.9 counts per glomerulus. This difference was statistically significant. Figure 1 shows the frequency distribution of glomeruli in different classes after semiquantitative analysis of mesangial carbon content of glomeruli of the sham-operated and the uninephrectomized rats. Whereas 60% of the glomeruli in the control group belong to the lowest class and none scores higher than 151 to 180 counts, a considerable percentage of glomeruli in the nephrectomized group shows mesangial carbon in the class of 181 to 210 counts and over.

Figure 2 (left side) shows a comparison of the frequency distribution of carbon content of normal glomeruli in control rats, normal glomeruli of experimental rats and diseased glomeruli in these same experimental animals. In the group of diseased glomeruli a significantly higher percentage belonged to

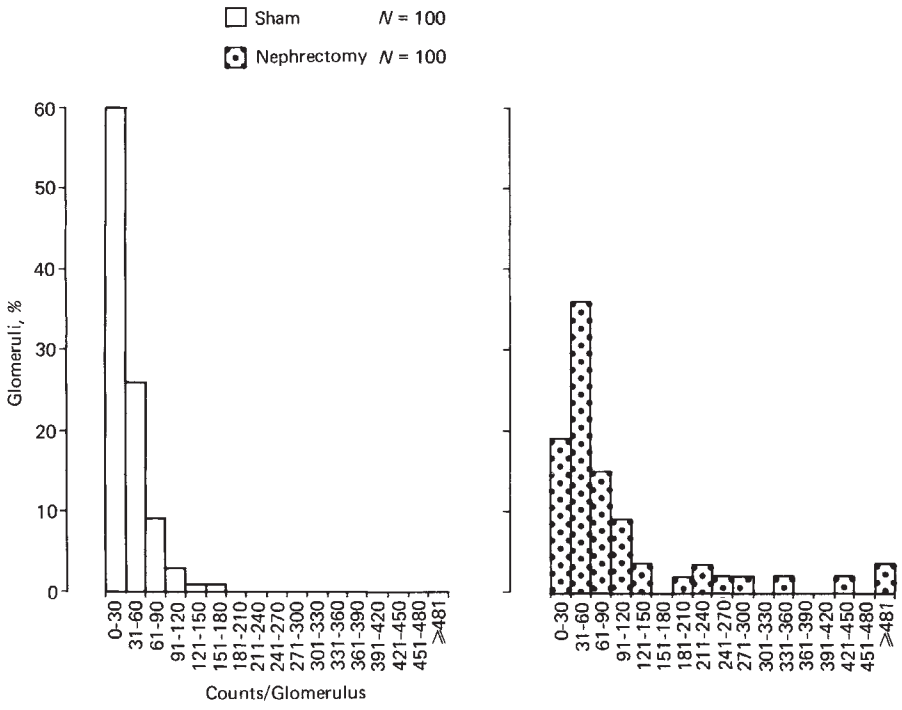

Fig. 1. *Frequency distribution of glomeruli in different classes of carbon counts in experiment A (carbon injected* after *unilateral nephrectomy/sham operation and 4 months before sacrifice).*

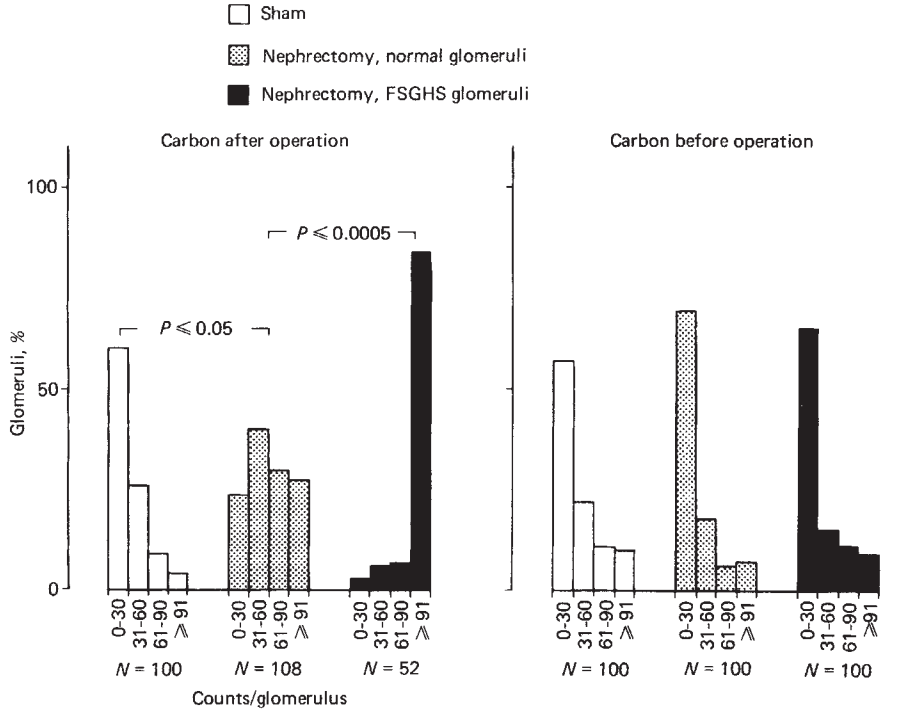

Fig. 2. *Frequency distribution of normal glomeruli of control and normal and diseased glomeruli of nephrectomized rats in the different classes of carbon count when carbon is injected* after *operation (experiment A, left side) and* before *operation (experiment B, right side).* Rats were sacrificed 4 months later. *P* values refer to differences in frequency distribution (Mann-Whitney U test).

the count class of 91 and over as compared to the group of normal glomeruli in the same kidneys and to the group of glomeruli of control rats. Compared to the glomeruli of control rats in the group of normal glomeruli of nephrectomized rats a significantly higher percentage belonged to the count class of 61 to 90 and 91 and over. Mean counts for normal and diseased glomeruli of the nephrectomized rats were 60.9 counts per glomerulus and 187.4 counts per glomerulus, respectively. Light microscopic examination showed that in the diseased glomeruli the carbon was localized perferentially *in* the lesions (Fig. 3A).

Experiment B. Urinary protein excretion in nephrectomized rats after 4 months was 48.2 ± 10.4 mg/24 hr and in sham-operated rats 10.7 ± 1.8 mg/24 hr. This difference was signifi-

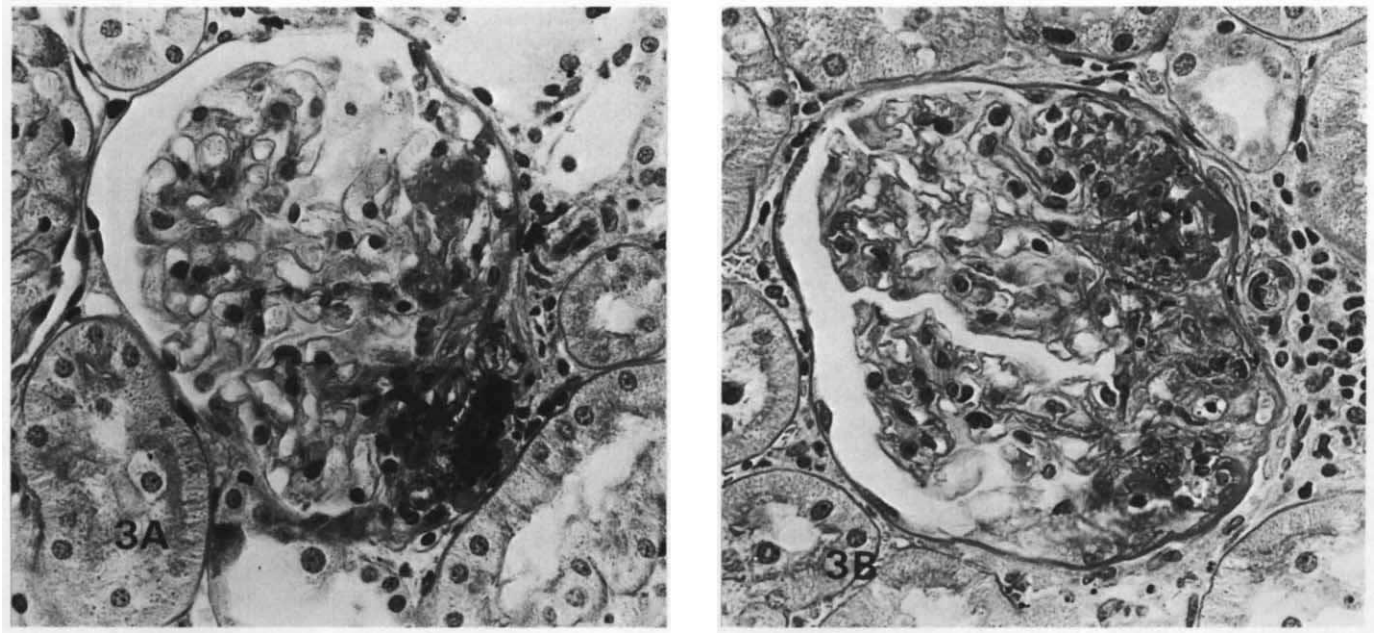

Fig. 3. A *Representative glomerulus of a rat which underwent unilateral nephrectomy* before *receiving i.v. injections of carbon (experiment A).* Large accumulations of carbon are present within an area with hyalinosis and sclerosis *(arrow).* (Periodic acid-Schiff, ×250) **B** *Glomerulus of a rat which underwent nephrectomy 48 hr* after *the last carbon injection (experiment B).* No localization of carbon is seen in the lesions. (Periodic acid-Schiff, ×250)

cant. At sacrifice 12.6 ± 4.1% of the glomeruli in the remaining kidney of the nephrectomized rats showed FSGHS. Compared to experiment A the percentage of glomeruli with FSGHS after 4 months was somewhat higher but the difference was not significant. No FSGHS lesions were found in the control group. Mean carbon content of glomeruli in the control group was 33.2 counts and in the unilateral nephrectomized group 28.9 counts per glomerulus. Mean counts for normal and diseased glomeruli in the nephrectomized rats were 28.6 and 29.7 counts per glomerulus, respectively. In contrast with experiment A the distribution over the different count classes of normal and diseased glomeruli in the remaining kidney of the nephrectomized rats and of glomeruli in sham-operated controls did not differ significantly (Fig. 2, right side). Moreover, these glomerular distributions were similar to that of the control rats of experiment A. Also, in contrast with the findings of experiment A no preferential localization of the remaining carbon in FSGHS lesions was found by light microscopic examination. In all three types of glomeruli the small amount of carbon present was seen mainly in the stalk area and at the vascular pole of the glomeruli. Figure 3**B** shows a representative glomerulus with a FSGHS lesion from experiment B.

Discussion

The most important observation from our experiments is the preferential localization of colloidal carbon in FSGHS lesions in experiment A when unilateral nephrectomy is performed *before* colloidal carbon is administered. Since the findings in experiment B indicate that unilateral nephrectomy does not result in decreased mesangial clearance, the accumulation of colloidal carbon in FSGHS lesions in experiment A must be due to increased uptake of carbon in areas where FSGHS is going to develop. As glomerular transcapillary hydrostatic pressure, glomerular blood flow, and glomerular filtration rate increase in the remaining kidney after unilateral nephrectomy [17, 21, 22], it is very likely that this increased uptake is caused by increased delivery of carbon to the glomerular mesangium.

It is quite clear from experiment A that the distribution of carbon among the normal and diseased glomeruli of nephrectomized rats differs considerably and that, probably the increased uptake of intravenously injected carbon by glomeruli in the remaining kidney after nephrectomy, therefore, is not *uniformly* distributed over all glomeruli. When the uptake of carbon and its distribution over the glomerular population is studied early after injection, it is found that in the nephrectomized rats the pattern of distribution frequency of the different classes of glomeruli according to carbon content is different as compared to that in two-kidney rats. In uninephrectomized rats both glomeruli ranging in similar classes as found in sham-operated rats and glomeruli with high carbon content are found (unpublished observations). The reason for this uneven distribution, possibly reflecting uneven glomerular reaction to changes in hemodynamic circumstances after nephrectomy, remains unclear. In the nephrectomized rats increased glomerular carbon uptake was not related especially to a subcapsular or juxtaglomerular position of the nephrons concerned.

It is excluded that the presence of increased amounts of carbon in the glomeruli is responsible for the development of FSGHS lesions because an even somewhat higher incidence of FSGHS was found in experiment B. Therefore, it is likely that both the increased uptake of carbon early in the experiment and the later development of FSGHS lesions are related to a common factor. The fact that carbon is localized especially in the lesions is a strong argument in favor of this hypothesis.

Although the increased uptake of carbon in glomeruli in experiment A may be caused by an increased flow, this does not explain the segmental character of the carbon deposition and the FSGHS lesion. We have drawn attention to the spatial relationship of the lesion with the afferent arteriole [4]. It may be that a high pressure at this point explains both the localization of carbon and the FSGHS lesion.

It has been suggested that disturbed function or overload of the mesangium may lead to sclerosis and scarring [3, 4, 10, 11, 17]. In these experiments we have given strong evidence for the latter possibility. As we excluded the possibility that carbon itself induces the lesions and made it likely that its presence only demonstrates the existence of increased uptake in apparently vulnerable areas, other as yet unknown substances deposited from the circulation must be responsible for the damage to the glomerulus.

Aging rats or unilaterally nephrectomized rats which develop FSGHS lesions already have increased urinary protein loss before the lesions have developed [3, 4, 6], and this is even clearer in rats made proteinuric by injections of aminonucleoside [23]. These experimental situations are characterized also by increased serum lipid levels [6, 7, 24], and fat is often present in FSGHS lesions in these situations [4, 6, 13]. The possibility that the deposition of lipid plays an important role in the pathogenesis of FSGHS lesions is an attractive one when it is remembered that mesangial cells represent modified smooth muscle cells [19, 25, 26]. It is well known from studies on the pathogenesis of atherosclerosis that the smooth muscle cell reacts with the incorporation of fat and deposition of different types of collagen and glycosaminoglycans to continuous exposure to cholesterol [27, 28]. We suggest, therefore, that both local hemodynamic factors and deposition of circulating substances such as lipids, may play a crucial role in the pathogenesis of FSGHS lesions.

Several authors have commented that FSGHS lesions in rats bear a striking resemblance to the lesions in patients with idiopathic focal sclerosis [3–5, 23]. It is now well recognized that these lesions by themselves do not represent a specific disease entity but rather a type of secondary phenomenon [29]. FSGHS lesions have been found in a wide range of kidney diseases such as membranous glomerulopathy [30], minimal lesions [31], reflux nephropathy [32], and heroin nephropathy [33]. It is thus quite clear that experimental models in the rat cannot be simply extrapolated to certain human kidney diseases. The similarity of the histologic picture in rat and man, however, is so striking that it seems worthwhile to further exploit the usefulness of the experimental model for obtaining more insight in the pathogenesis of FSGHS lesions in man.

Acknowledgments

A portion of this work was presented at the 18th meeting of the Dutch Society of Nephrology, Amsterdam, November 15, 1980, and has been published in abstract form (*Kidney Int* 20:313, 1981). This work was supported by the Dutch Kidney Foundation. We thank Dr. N. Lameire, Department of Nephrology, University of Ghent, Ghent, Belgium, for critical review of the manuscript, Miss C. Mein for technical assistance, and Miss J. Kop for her help in preparing the manuscript.

Reprint requests to Dr. J. Grond, Department of Pathology, University of Groningen, Oostersingel 63, 9713 EZ Groningen, The Netherlands

References

1. Rich AR: A hitherto undescribed vulnerability of the juxtamedullary glomeruli in lipoid nephrosis. *Bull Johns Hopkins Hosp* 100:173–186, 1957
2. Habib R: Focal glomerular sclerosis. *Kidney Int* 4:355–361, 1973
3. Couser WG, Stilmant MM: Mesangial lesions and focal glomerular sclerosis in the aging rat. *Lab Invest* 33:491–501, 1975
4. Elema JD, Arends A: Focal and segmental glomerular hyalinosis and sclerosis in the rat. *Lab Invest* 33:554–561, 1975
5. Kreisberg JI, Karnovsky MJ: Focal glomerular sclerosis in the Fawn-Hooded rat. *Am J Pathol* 92:637–645, 1978
6. Schilthuis MS: Focale segmentale glomerulaire hyalinose en sclerose. Een klinisch-pathologisch en experimenteel onderzoek. Academic Thesis, University of Groningen, 1979
7. Berg BN: Spontaneous nephrosis with proteinuria, hyperglobulinaemia, and hypercholesterolaemia in the rat. *Proc Soc Exp Biol Med* 119:417–420, 1965
8. Blatherwick NR, Medlar EM: Chronic nephritis in rats fed high protein diets. *Arch Intern Med* 59:572–596, 1937
9. Elema JD, Koudstaal J, Lamberts HB, Arends A: Spontaneous glomerulosclerosis in the rat. *Arch Pathol Lab Med* 91:418–425, 1971
10. Elema JD, Arends A: Functional overload as a possible cause for non-immune glomerular damage. Effect of nephrectomy and irradiation of the contralateral kidney. *J Pathol* 103:21–28, 1971
11. Lalich JJ, Faith GC, Harding GE: Protein overload nephropathy. *Arch Pathol Lab Med* 89:548–554, 1970
12. Lalich JJ, Allen JR: Protein overload nephropathy in rats with unilateral nephrectomy. II. Ultrastructural study. *Arch Pathol Lab Med* 91:372–382, 1971
13. Lalich JJ, Burkholder PM, Paik WCW: Protein overload nephropathy in rats with unilateral nephrectomy. A correlative light, immunofluorescence and electron microscopic analysis. *Arch Pathol Lab Med* 99:72–79, 1975
14. Bolton WK, Benton BS, Maclay BA, Sturgill BC: Spontaneous glomerulosclerosis in aging Sprague-Dawley rats. I. Lesions associated with mesangial IgM deposits. *Am J Pathol* 85:277–302, 1977
15. Bolton WK, Sturgill BC: Spontaneous glomerular sclerosis in aging Sprague-Dawley rats. II. Ultrastructural studies. *Am J Pathol* 98:339–356, 1980
16. Sellers AL, Goodman HC, Marmoston J: Sex difference in proteinuria in the rat. *Am J Physiol* 163:662–667, 1950
17. Olson JL, Hostetter TH, Rennke HG, Brenner BM: Altered charge and size selective properties of the glomerular wall: A response to reduced renal mass (*abstract*). *Kidney Int* 16:857, 1979
18. Elema JD, Hoyer JR, Vernier RL: The glomerular mesangium: Uptake and transport of intravenously injected colloidal carbon in rats. *Kidney Int* 9:395–406, 1976
19. Grond J, Daha MR, Elema JD: Glomerular mesangial uptake of intravenously injected colloidal carbon in the rat determined with an image analysing method: Lack of influence of platelet aggregation or complement activation. *Virchows Arch [Cell Pathol]* 33:281–291, 1980
20. Weibel ER: Stereological principles for morphometry in electron microscopic cytology. *Int Rev Cytol* 26:235–302, 1969
21. Azar S, Johnson MA, Hertel D, Tobian L: Single nephron pressures, flows, and resistances in hypertensive kidneys with nephrosclerosis. *Kidney Int* 12:28–40, 1977
22. Deen WM, Maddox DA, Robertson CR, Brenner BM: Dynamics of glomerular ultrafiltration in the rat. VII. Response to reduced renal mass. *Am J Physiol* 227:556–562, 1974
23. Glasser RJ, Velosa JA, Michael AF: Experimental model of focal sclerosis. I. Relationship to protein excretion in aminonucleoside nephrosis. *Lab Invest* 36:519–526, 1977
24. Hoyer JR, Mauer SM, Michael AF: Unilateral renal disease in the rat. I. Clinical, morphologic, and glomerular mesangial functional features of the experimental model produced by renal perfusion with aminonucleoside. *J Lab Clin Med* 85:756–768, 1975
25. Michielsen P, Creemers J: The structure and function of the glomerular mesangium, in *Ultrastructure of the Kidney*, edited by Dalton AJ, Haguenau F, New York and London, Academic Press, 1967, p. 57

26. AUSIELLO DA, KREISBURG JI, ROY C, KARNOVSKY MJ: Contraction of cultured rat glomerular cells of apparently mesangial origin after stimulation with angiotensin II and arginine vasopressin. *J Clin Invest* 65:754–760, 1980
27. ROSS R, GLOMSET JA: The pathogenesis of atherosclerosis. I. *N Engl J Med* 295:369–377, 1976
28. ROSS R, GLOMSET JA: The pathogenesis of atherosclerosis. II. *N Engl J Med* 295:420–426, 1976
29. BALDWIN DS: Chronic glomerulonephritis: Nonimmunologic mechanisms of progressive glomerular damage. *Kidney Int* 21:109–120, 1982
30. KINCAID-SMITH P: *The Kidney. A Clinico-Pathologic Study*. Oxford, Blackwell Scientific Publications, 1975, p. 129
31. ROSEN S, GALVANEK E, LEVY M, HABIB R: Glomerular disease. *Hum Pathol* 12:964–977, 1981
32. BATHENA DB, WEISS JH, HOLLAND NH, MCMORROW RG, CURTIS JJ, LUCAS BA, LUKE RG: Focal and segmental glomerular sclerosis in reflux nephropathy. *Am J Med* 68:886–892, 1980
33. GRISHMAN E, CHURG J, PORUSH JC: Glomerular morphology in nephrotic heroin addicts. *Lab Invest* 35:415–424, 1976